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JC Virus DNA Is Present in Many Human Brain Samples from Patients without Progressive Multifocal Leukoencephalopathy

FRANK A. WHITE III,[1]† MOHAMMAD ISHAQ,[2] GERALD L. STONER,[2] AND RICHARD J. FRISQUE[1]*

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802,[1] and Laboratory of Experimental Neuropathology, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland 20892[2]

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Sections of normal and diseased brain and kidney tissues were screened for the presence of JC virus (JCV) DNA by using the polymerase chain reaction. As expected, all samples obtained from patients with progressive multifocal leukoencephalopathy (PML) tested positive when multiple JCV-specific primer and probe combinations were used. Unexpectedly, more than 50% of non-PML-affected brains were also found to harbor low levels of JCV DNA. To confirm that the positive signals seen in the tissue sections were not the result of contamination, amplified DNA was cloned and sequenced and in some cases was shown to represent strains of JCV not identified previously. Two predominant regulatory region configurations of JCV have been detected in the human host: archetype JCV, which is excreted in the urine of normal and immunocompromised individuals, and "PML-type" JCV found in diseased brains. This latter group of variants appears to derive from archetype JCV by the deletion and duplication of sequences within the promoter-enhancer region. In the present study, the archetype strain of JCV was identified only in normal kidney samples; JCV DNA found in non-PML-affected brain specimens and in kidney tissue from patients with PML resembled that of strains isolated from PML-affected brain tissue. Our findings indicate that JCV reaches the brain more frequently than previously thought and may persist at this site without causing demyelinating disease. A subsequent episode of prolonged immunodeficiency or a direct interaction with an immunocompromising agent (e.g., human immunodeficiency virus type 1) might activate the latent JCV infection and lead to the development of PML.

The polyomavirus JC virus (JCV) infects some 70% of the adult population (53, 63) and is the etiologic agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (54). Once considered a rare illness that occurred most frequently in elderly individuals as a terminal complication of lymphoproliferative disease (8), the incidence of PML has increased dramatically in recent years as a result of the AIDS epidemic (6). Impairment of immune function by human immunodeficiency virus type 1 (HIV-1) infection contributes to the pathogenesis of PML, but additional factors, such as the ability of the HIV-1 *tat* protein to activate JCV transcription (62) and the potential for HIV-1 to infect brains which may already harbor JCV (51, 67, 68), may help explain the relatively high incidence of PML (2 to 7%; 2, 35, 39, 48, 55, 61) in patients with AIDS.

It has been suggested that JCV circulating in the population might persist in the kidney in a form different from that which causes PML (11, 14, 20, 44). PML is thought to generally arise following reactivation of a persistent kidney infection (reviewed in reference 21). During such an event, alterations to the kidney strain of JCV could generate a variant capable of infecting and lysing oligodendrocytes in the brain. Results from several laboratories support this latter possibility (14, 44, 45) and indicate that one type of JCV, termed archetype (72), is predominant in the urine of immunocompromised and pregnant individuals (17, 43, 71). During prolonged immune suppression, new strains of JCV are detected in the urine; these strains differ from archetype by deletions and duplications within the promoter-enhancer region (50, 71). These variants resemble virus found in PML-affected brain tissue.

JCV DNA has been detected in several organs of patients with PML, including kidney, spleen, and lung (10, 16, 27, 32). In non-PML-affected patients, the JCV genome has been found in approximately 10% of normal kidney samples (10) and in the urine of immunodeficient individuals (3, 4, 13, 17, 22, 34, 50, 71). Attempts to uncover JCV in non-PML-affected brain tissue by using immunofluorescence and DNA hybridization methods have met with little success. These studies have failed to detect JCV in cells cultured from brain tumors (24) or in the brains of patients with schizophrenia (9) or multiple sclerosis (46). Dörries and coworkers (15) found BK virus (BKV) but not JCV or simian virus 40 (SV40) DNA in human brain tumors when they used specific viral DNA probes. JCV DNA was discovered in biopsy and postmortem samples from the brains of eight patients with neurological diseases, but seven of these showed clinical or laboratory evidence of PML (23). Recently, Mori et al. (47) found JCV DNA and late proteins in the brain glial cells of 3 of 10 elderly patients (68 to 96 years old) without known neurological disease but not in those of 4 younger individuals (32 to 57 years old). However, conventional histology revealed small demyelinated foci suggestive of subclinical PML in the three elderly patients.

The development of the polymerase chain reaction (PCR) (49, 57) has led to the ability to identify the presence of DNA in tissues at levels previously undetectable. Despite potential problems associated with its high sensitivity (40), PCR has been successfully applied to the detection and diagnosis of several viral infections involving HIV (38, 52), human

* Corresponding author.
† Present address: SRA Technologies Inc., Rockville, MD 20850.

papillomavirus (60, 66), and hepatitis B virus (33). Recently, PCR has been used to detect and characterize JCV DNA in urine (3, 17) and in formalin-fixed, paraffin-embedded PML-affected brain samples (64). However, JCV DNA has not been found in either fixed (64) or unfixed (31) specimens of control (non-PML-affected) brain tissue.

We now report the detection and characterization of JCV DNA in frozen brain samples from individuals who had not been diagnosed as having PML and who lacked pathological evidence of demyelinating disease.

MATERIALS AND METHODS

PCR precautions and optimization. False positives, caused by contamination or by amplification of related sequences, are a well-documented problem of PCR analyses (37, 40). To reduce the chance of contamination, the following precautions were taken. All reagents were made from fresh stock chemicals and stored in sterile disposable plastic containers. PCR buffer, nucleotides, and primers were prepared in quantity, checked for contamination, and stored in single-use aliquots. A master mix containing these reagents and *Taq* polymerase was prepared and aliquoted. Reagents, tissues, and PCR mixture were prepared in laminar-flow hoods or biological safety cabinets in separate rooms. For each set of PCR, multiple negative controls were prepared. These included reactions in which (i) all but one component (e.g., tissue extract, primers, or polymerase) were present in the tube, (ii) BKV or SV40 DNA was used as template, or (iii) extracts of normal hamster brain tissue were tested. Reagents for the negative controls were aliquoted after each set of tissues was prepared, and one such control was used for every six human samples analyzed. Positive controls containing recombinant JCV DNA were prepared only after all tissue sample tubes had been sealed. Separate dedicated sets of positive-displacement pipettors (Gilson, Villiers-le-Bel, France) were used for preparation of stock reagents and for individual amplification reactions. Microtomes, the thermal cycler, tube racks, and pipettors were cleaned with bleach or 0.1 to 1 M HCl and in some cases subjected to UV illumination between experiments.

To determine whether contamination of tissue samples occurred before the PCR was conducted (65), a set of primers and probe that would identify the recombinant clone, pMad1-TC, was employed (20). This recombinant clone is the form of JCV DNA used most frequently in our laboratories; pMad1-TC is also the source of a commercial JCV probe used in some diagnostic laboratories. The primer pair (JJP 1 and pJP 2; Table 1) amplifies a DNA segment that spans a unique *Eco*RI site joining the JCV and pBR322 sequences. Amplification of this 313-bp virus-plasmid DNA product from a tissue sample would unequivocally identify JCV contamination from a laboratory source.

To increase the sensitivity and specificity of the PCR, the following criteria were tested and optimized. Sequences of each primer were selected to differentiate between the closely related polyomaviruses, BKV, SV40, and JCV. Pairs of primers were designed to amplify sequences from the early (T-antigen), late (VP1), and regulatory regions of the JCV genome (Table 1). The last pair of primers was used in the identification, cloning, and sequencing of variant genomes present in tissue. The ratios and total quantities of the two primers in each pair were optimized to yield the greatest amount of specific product with the least amount of "primer-dimer" artifact. Annealing temperatures were selected to ensure specificity of the amplification and discrimination between the closely related viruses; these temperatures were 3 to 8°C below the T_m of each primer. The final Mg^{2+} concentration was found to be optimal within a broad range of 1.0 to 5.0 mM and was set at 1.5 mM for all experiments. Recombinant AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was used in these studies, since it gave slightly greater specific yields than purified *Taq* polymerase (Perkin-Elmer Cetus or Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

TABLE 1. PCR oligonucleotide primers and probes

Oligonucleotide name[a]	Sequence (5'→3')[b]	Nucleotide positions[c]
JEP 1	CCTGTGTGTCTGCACCAGAGGC	2674–2695
JEP 1.1	CAGTGCTTGATCCATGTCCAGAGTC	2731–2755
JEP 2	GGCCAGTTGCTGACTTTGCAGC	2919–2898
JLP 1	ACATGTGGGAGGCTGTGACCT	1771–1790
JLP 1.1	AGCCAGTGCAGGGCACCAGC	1872–1891
JLP 2	CCTAGGTACGCCTTGTGCTCTG	2039–2019
JRR 1	CCTCCACGCCCTTACTACTTCTGAG	5086–5110
JRR 1.1	CCTAGGGAGCCAACCAGCTAACAGC	164–188
JRR 2	GTGACAGCTGGCGAAGAACCATGGC	298–274
JJP 1	AGAAATGGGTGACCCAGATGAG	1606–1627
JJP 1.1	ACATGCTTCCTTGTTACAGTGTGGC	1695–1719
pJP 2	ATGGACGATATCCCGCAAGAGG	196–175
BEP 3	CACAGCAAAGCAGGCAAGGGTTC	4213–4235
BEP 3.1	CTGGTGTAGATCAGAGGGAAAGTC	4264–4287
BEP 4	GGGAGTCCTGGTGGAGTTCC	4433–4414

[a] The first letter (J, B, or p) of each name represents JCV, BKV, or pBR322, respectively. The next two letters (EP, LP, RR, or JP) indicate early-, late-, regulatory-, or junction region primers and probes. Whole numbers refer to primers, and numbers with decimals are probes. A set of two primers and one probe is used for PCR amplification and detection.

[b] The A and C at the 5' ends of primers JLP 1 and JLP 2, respectively, do not represent JCV nucleotide sequence.

[c] Nucleotide numbering for JCV and BKV is from Frisque et al. (20) and Seif et al. (59), respectively.

Human brain tissues. All brain sections included white matter. Samples identified by letters (see Table 2) were cerebral cortex with subcortical white matter, except for AE and OR, which were only white matter. Samples identified by numbers were from white-matter areas in the cerebral hemispheres, except for 929, which contained cerebellar cortex with underlying white matter. PML- and non-PML-affected tissues were received from different sources at different times and handled on different days. All specimens were individually wrapped in plastic, sealed in plastic bags, and kept in separate boxes in a freezer at −70°C. Materials coming in contact with individual specimens during sectioning were discarded (gloves) or sterilized (tools, e.g., forceps) before a second sample was dealt with. Tissue sections were prepared at one institution (National Institutes of Health, laboratory of G.L.S.) and shipped as 10-μm-thick frozen sections in sterile Eppendorf tubes to a second institution (Pennsylvania State University, laboratory of R.J.F.), where the PCR were conducted. Non-PML-affected tissues were cut with disposable blades in a cryostat located on a different floor from the cryostat used to cut PML-affected tissues. No PML-affected tissues or JCV stocks were stored or handled in the room in which non-PML-affected tissues were handled. Tissues were suspended in 100 μl of digestion buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 0.45% (vol/vol) Tween 20, 0.45% (vol/vol) Nonidet P-40, and 10 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.); vortexed thoroughly; and incubated for 75 min at 56°C. Proteinase K was inactivated by incubation at 95°C for 15 min. Samples were pelleted for 5 min in a microcentrifuge and frozen at −20°C until analyzed.

PCR conditions. PCR was carried out in a total volume of 50 μl with final concentrations of 50 mM KCl; 10 mM Tris (pH 8.3); 1.5 mM $MgCl_2$; 0.001% gelatin; 200 μM each dATP, dCTP, dGTP, and dTTP; 2.0 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus); and 100 to 250 pmol of each primer (sequences for each primer pair used are listed in Table 1). Each reaction mixture was overlaid with 40 μl of sterile mineral oil. Cycling parameters in a Perkin-Elmer Cetus DNA Thermal Cycler were 94°C for 45 s (denaturation), 58°C for 30 s (annealing), and 72°C for 60 s (extension) for 40 cycles, with an additional 5 min added to the extension step during the last cycle. Positive controls contained 10 to 1,000 molecules of pMad1-TC (prototype Mad1 genome of JCV cloned into the *Eco*RI site of pBR322; 20) and were prepared after the tissue sample reaction tubes had been closed. Negative controls are described above.

PCR product analysis. One-fifth of each reaction mixture (10 μl) was electrophoresed on a 2% NuSieve (FMC Bioproducts, Rockland, Maine)–0.7% agarose (molecular-biology grade; IBI Biotechnologies, New Haven, Conn.) gel containing 0.5 μg of ethidium bromide per ml in 1× TBE buffer (90 mM Tris [pH 8.3], 90 mM sodium borate, 2.5 mM EDTA). Electrophoresis was conducted at 140 V for 50 min with a Hoeffer minisubmarine gel apparatus. Following electrophoresis, gels were photographed and then prepared for Southern transfer by treating them for a minimum of 45 min in 0.2 N NaOH–0.6 M NaCl and then for a minimum of 45 min in 1.0 M Tris (pH 7.4)–0.6 M NaCl. Transfer of DNA to Nytran membranes (Schleicher & Schuell, Keene, N.H.) was accomplished by blotting for 16 h or more in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate). DNA was UV cross-linked to the filters by using 254-nm germicidal lamps generating a UV dosage of 1.6 kJ/m^2 (12). Filters were prehybridized in 5× Denhardt's solution (20 μg each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone per ml) and 6× SSC at 58°C for at least 1 h. The solution was replaced with a fresh hybridization solution of 5× Denhardt's, 6× SSC, and 0.5% sodium dodecyl sulfate (SDS) containing approximately 300,000 cpm of [^{32}P]ATP-end-labeled oligonucleotide probe (sequences listed in Table 1) for each filter. Hybridization was done at 58°C for a minimum of 5 h. Filters were washed by using the following conditions; 15 min in 2× SSC–1.0% SDS at room temperature, 15 min in 1× SSC–1.0% SDS at 58°C, and 15 min in 0.2× SSC–1.0% SDS at 58°C. Filters were exposed on Kodak X-AR film overnight at −70°C with a Dupont Cronex Lightning-Plus intensifying screen.

Recombinant DNA cloning. The cloning vector BSMSK-Nco was produced by ligating an oligonucleotide adapter (CTAGCCATGG) containing an *Nco*I restriction site into the *Spe*I site of Bluescript BSMSK.

Following identification of the tissue samples that contained JCV DNA, the remaining portion of the PCR mixture was purified on a Qiagen PCR purification column (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's suggested protocol. Samples were digested with *Hin*dIII (40 U) in buffer containing 50 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol for a minimum of 2 h at 37°C. The NaCl concentration was adjusted to 100 mM, and the samples were digested with *Nco*I (30 U) for an additional 2 h. The restriction endonucleases were heat inactivated at 56°C for 15 min, and the reaction mixture was desalted by using a Sephadex G-50 spin column equilibrated in TE (10 mM Tris [pH 8.0], 1 mM EDTA) and was precipitated by using 0.1 volume of sterile sodium acetate and 3 volumes of 100% ethanol. Following a 70% ethanol wash, samples were lyophilized, suspended in 10 μl of sterile H_2O, and ligated to 50 ng of *Hin*dIII-*Nco*I-digested BSMSK-Nco for 18 h at 15°C. *Escherichia coli* DH1 was transformed with 10% of the ligation mixture by using the protocol of Hanahan (29), and transformants were selected for ampicillin resistance. Colony lifts and hybridizations were done by the procedure of Grunstein and Hogness (28), and transformants containing a JCV regulatory region were identified by hybridization with a ^{32}P-end-labeled oligonucleotide probe by using the procedures described above, with the following changes. Probes were hybridized overnight at 55°C in 5× Denhardt's–6× SSC, and filters were washed for 15 min in 1× SSC–0.1% SDS at room temperature and then twice for 15 min per wash in 1× SSC–0.1% SDS at 55°C. Positive colonies were detected after autoradiography (2 to 4 days at −70°C) with Fuji RX film with a Dupont Cronex Lightning-Plus intensifying screen. Recombinant DNA was prepared from these colonies by alkaline lysis (5) and digested with *Xba*I and *Xho*I to confirm the presence of an insert. In the absence of an insert, this digestion yielded a 63-bp fragment from the polylinker region of BSMSK-Nco.

DNA sequencing. All clones containing a JCV DNA insert were prepared by the alkaline lysis method, suspended in 50 μl of TE, and sequenced (5 μl) by using the following modifications of the dideoxy protocol (58). Reactions were carried out at 42°C using T_7 and T_3 sequencing primers (Stratagene, La Jolla, Calif.), [^{35}S]dATP (New England Nuclear, Boston, Mass.), 7-deaza-dGTP (in place of dGTP), *E. coli* single-strand binding protein (final concentration, 150 μM; gift from Ken Johnson), and Klenow reagent (3 U; sequencing grade; Boehringer Mannheim). Reaction products were electrophoresed for 2 or 5 h in 0.6× TBE running buffer on Long-Ranger Hydro-Link denaturing acrylamide gels (AT Biochem, Malvern, Pa.) prepared with 1.2× TBE. Gels were dried and exposed to Fuji RX film for 14 to 48 h without an intensifying screen. Sequences were determined for both strands of each recombinant DNA insert and were analyzed on an IBM XT computer by using IBI Pustell DNA analysis software.

Immunocytochemistry. Brain sections were examined for expression of the JCV early-protein T antigen by using monoclonal antibody PAb 1614 and a slight modification of the immunoperoxidase technique previously described (56). The positive control consisted of known PML-affected tissue.

In situ hybridization. A biotinylated JCV DNA probe supplied by Enzo Diagnostics was used according to the manufacturer's instructions and published procedures (7). The detection system consisted of streptavidin-peroxidase (1:100) (Kirkegaard and Perry Laboratories) applied for 1 h at room temperature followed by washing and treatment with the substrate 3,3′-diaminobenzidine enhanced with $NiCl_2$ for 10 min at room temperature (56). The positive control consisted of known PML-affected tissue.

Histology. Tissue sections were examined for pathological changes following staining with hematoxylin-eosin.

RESULTS

To detect the presence of JCV DNA in various tissues, 10-μm-thick sections of frozen brain and kidney tissues from PML- and non-PML-affected patients (Table 2) were subjected to PCR analysis in several independent experiments. Multiple specific primer and probe combinations were em-

TABLE 2. Data for patients without PML whose tissues were used for PCR experiments

Code	Age (yr)/sex	Diagnosis	No. of positives/no. of reactions[a]	
			JCV	JCV-pBR322[b]
AE	70/M	Squamous cell CA[c]	6/16 (12)	0/3 (1)
AEL	65/M	Myocardial infarct	1/3 (2)	NT
HJ	89/M	Pneumonia, cancer	0/3 (2)	NT
HM	73/M	Pulmonary embolism	0/3 (3)	NT
KG	63/M	Respiratory failure	3/11 (9)	0/3 (1)
LL	50/M	Cardiac arrest	3/11 (6)	0/5 (2)
LV	57/M	*Pseudomonas* infection	5/9 (5)	0/5 (2)
MA	64/M	Severe cirrhosis	5/12 (8)	0/3 (1)
ME	93/M	Systemic atherosclerosis	2/6 (4)	NT
MED	69/M	Cardiopulmonary disease	1/8 (6)	0/3 (1)
MO	56/M	Myocardial infarct	1/10 (8)	0/3 (1)
OR	64/M	Myocardial infarct	6/13 (10)	0/3 (1)
OV	34/M	Cardiac arrest, adeno-CA	2/16 (12)	0/3 (1)
PJ	57/M	Heart failure, cancer	4/12 (8)	NT
RA	69/M	Heart failure	5/17 (11)	0/5 (2)
RL	65/M	Oat cell CA	5/12 (8)	0/3 (1)
736	41/F	Auto accident	4/10 (6)	2/3 (1)
884	86/M	Tourette's syndrome	7/17 (10)	0/4 (2)
904	58/M	Lymphoma	8/15 (10)	0/5 (2)
929	61/M	Pneumonia	12/18 (10)	0/11 (5)
938	43/F	Metastatic breast CA	11/14 (7)	0/5 (2)
982	78/M	Alzheimer's disease	0/2 (1)	NT

[a] The number of contiguous brain sections tested is given in parentheses. A single tissue section was usually examined one or two times.
[b] To rule out the possibility of contamination of the brain samples with cloned JCV DNA, PCR amplification and detection of a JCV-pBR322 junction fragment was attempted by using the JJP 1 and pJP 2 primers and JJP 1.1 probe. These primers amplified positive-control samples at least as well as the JCV-specific primers. Evidence of contamination was found only in specimen 736; a faint band was detected in two of the three reactions. NT, not tested.
[c] CA, carcinoma.

ployed (Table 1) to ensure that a positive signal was not the result of amplification of cellular sequences or of DNA from a related virus (SV40 or BKV). All JCV-specific primers amplified fewer than 10 molecules of recombinant JCV DNA to levels detectable by Southern blotting, although the late-region primer-probe combination was at least 10-fold less sensitive than combinations representing the early and regulatory regions. Amplification was not observed with any of these primers when 1,000 molecules of the SV40 or BKV genomes were used as the target DNA (data not shown).

The extreme sensitivity of the PCR method makes the possibility of false-positive results a very real concern. Extensive precautions (detailed in Materials and Methods) were taken to reduce the possibility of contamination during tissue or reagent preparation. In the unusual event (<5%) that a negative control in a given set of reactions produced a positive signal for the viral DNA being tested, all results for that experiment were disregarded. As an additional negative control, sections of normal hamster brain were cut before and after the human brain samples were cut. This was done to examine the possibility of carryover contamination via the cryostat during the sectioning. These hamster brain sections were consistently negative for JCV DNA.

Samples of PML-affected brain tissue were the first to be tested for the presence of JCV DNA. As expected, the specific early-, late-, and regulatory-region primers amplified JCV DNA in all seven specimens (100%; Table 3). An autoradiogram representative of these results is shown in Fig. 1.

The results of the experiments with non-PML-affected brain samples are summarized in Table 3, and representative autoradiograms are shown in Fig. 2. When tested with primers and probes specific for the different regions of the JCV genome, tissue sections from 15 (68%) of 22 non-PML-affected brains were positive two or more times for JCV DNA. Unlike the PML-affected brain samples, however, not all sections of non-PML-affected brains gave a positive signal. Furthermore, the intensity of their signals was significantly less than that observed with the PML-affected tissues (compare Fig. 1 and 2).

The JCV DNA clone pMad1-TC is used routinely in our laboratories and represents a potential source of contamination during tissue handling. To rule out the possibility that pMad1-TC was responsible for the positive PCR results, attempts were made to amplify a JCV-pBR322 junction fragment with primers flanking the *Eco*RI cloning site in this recombinant DNA (Table 2). Evidence for contamination was found only in a single tissue section from patient 736; a weak band was detected in two of three reactions.

TABLE 3. Summary of PCR results

Tissue		No. of positives[a]/no. of reactions (%)
Type of patient	Organ	
PML	Brain	7/7 (100)
PML	Kidney	5/5 (100)
Non-PML	Brain	15/22 (68)[b]
Non-PML	Kidney	7/14 (50)[b]

[a] A sample was scored as positive when two or more preparations of that sample gave a detectable signal following Southern hybridization of the PCR product with a JCV-specific oligonucleotide probe. Because of the limitations of PCR analysis and the low level of signal detected with non-PML-affected specimens, this conservative approach to identifying a sample as JCV positive was considered appropriate.
[b] Differences in the percents positive in non-PML-affected kidney and brain tissues were not considered significant; slightly higher values for the brain specimens might be attributed to the greater ease with which DNA is extracted from this tissue relative to kidney tissue.

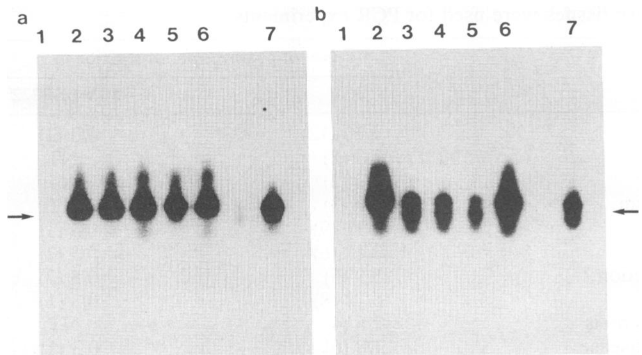

FIG. 1. Detection of JCV DNA in PML-affected brain and kidney tissues. JCV DNA was amplified with the JLP 1 and 2 primers and detected by hybridization with a ^{32}P-end-labeled oligonucleotide probe specific for sequences located between the two primers (JLP 1.1). Exposure to the film was for 18 h with an intensifying screen. Lane 1 in each panel is a negative control. The signal seen in the positive control (lane 7) is amplified from 1,000 molecules of recombinant JCV(Mad1) DNA. Arrows indicate positions of the amplified 271-bp fragments. Each remaining lane corresponds to tissue from a different patient with PML. (A) Brain samples from PA (lane 2), RY (lane 3), LE (lane 4), KO (lane 5), and BO (lane 6); (B) kidney samples from PA (lane 2), GU (lane 3), LE (lane 4), DA (lane 5), and PO (lane 6). All patients with PML, except GU, were diagnosed as having AIDS.

In addition to brain tissue samples, kidney tissues from PML- and non-PML-affected patients were also tested. Using the same combinations of primers and probes, JCV DNA was detected in 5 (100%) of 5 kidneys from patients with PML and in 7 (50%) of 14 kidneys from patients without PML (Fig. 1; Table 3). Again, more variability was seen with non-PML-affected samples; not all sections gave a positive signal, and fewer copies of the JCV genome were present.

While stringent measures were taken to avoid contamination, we reasoned that the presence of JCV in non-PML-affected brain would be demonstrated most convincingly by the cloning and sequencing of the DNA from several positive samples and confirming that at least some of these strains differed from those used in our laboratories. This approach would also allow us to identify JCV variants that might be present in the different tissues. Direct sequencing of the PCR products could produce ambiguous results if multiple variants were present in the samples. Therefore, the reaction products were cloned prior to sequence analysis. By omitting gel purification of the amplified DNA, the selection of a particular species of viral genome was avoided, thereby increasing the likelihood that the clones obtained would be representative of all the JCV species present in a given sample. Using the JRR 1 and 2 primers, DNA to be cloned was amplified from several brain and kidney samples of normal and PML-affected patients. These primers overlap sequences that are highly conserved in different strains of JCV and flank the regulatory region containing the DNA replication origin and the hypervariable promoter-enhancer sequences. It is this variation within the transcriptional control elements that has been used to distinguish strains of JCV isolated from different individuals (41, 45, 72). While most JCV variants obtained from different patients with PML have been unique, a few, including Mad1 and Mad8Br, have been isolated from more than one patient (26, 50).

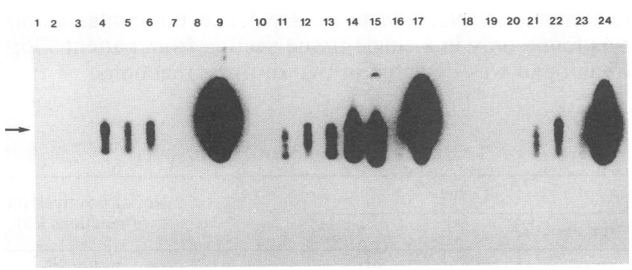

FIG. 2. Detection of JCV DNA in brain tissues from non-PML-affected patients. JCV DNA was amplified with the JRR 1 and 2 primers and detected by hybridization with a ^{32}P-end-labeled oligonucleotide probe specific for sequences located between the two primers (JRR 1.1). Exposure to the film was for 18 h with an intensifying screen. Lanes 1, 2, 10, 18, and 19 are negative controls, and lanes 9, 17, and 24 are positive controls containing 100 molecules of recombinant JCV(Mad1) DNA each. The arrow indicates the position of the amplified 343-bp fragment. Each remaining lane corresponds to tissue from a different patient. Lanes: 3, KG; 4, LV; 5, MA; 6, OR; 7, RA; 8, RL; 11, 736; 12, 884; 13, 904; 14, 929; 15, 938; 16, MED; 20, MO; 21, LL; 22, AE; 23, OV. Samples in lanes 4 through 6, 11 through 15, 21, and 22 contained JCV DNA.

The sequences of several regulatory regions cloned by this approach are shown in Fig. 3. Multiple clones from a single tissue sample were identical in most cases and differed from those recovered from tissues of other individuals. The clones obtained from non-PML-affected brain samples represented regulatory regions that differed from those found in JCV variants isolated in previous studies with the following exceptions: the clone obtained from the LV sample and two of three clones obtained from the 929 specimen were identical to JCV(Mad1). Clone 929-3, however, exhibited a unique deletion of 10 nucleotides encompassing the second (upstream) TATA box. This deletion resulted in the generation of an enhancer composed of 100-bp repeats that did not include the remaining TATA signal. This arrangement resembles the regulatory region of the Mad4 variant of JCV (45). The remaining clones from non-PML-affected brain tissues represented novel JCV variants. A single clone from patient OV was a type II variant (45) exhibiting a single TATA box, the 23-bp conserved block, and 66-bp nontandem repeat elements. Two clones from patient 938 had a single TATA box, the 23-bp block, and 62-bp tandem repeats.

Sequence analysis of multiple clones obtained from the kidney tissues of two patients without PML (patients 482 and 552) identified the only archetype regulatory regions uncovered in this study (Fig. 3). The two sets of clones differed from one another at four nucleotide positions; two of these positions have been identified as hot spots of variation in the archetype regulatory region (72).

Brain tissue samples from two patients with PML (BO and RY) yielded novel type II variant clones containing 1 TATA box, the 23-bp block, and 82- and 76-bp tandem repeats, respectively. Clones obtained from a third patient (GU) were identical to Mad8Br, a variant isolated previously from two other patients (45, 50).

Six regulatory-region clones were obtained from the kidneys of two patients with PML, and unlike those from normal kidneys, all had novel type II rather than archetype regulatory sequences. Clones derived from the first patient (PO) contained two sets of nontandem repeats of 50 and 12 bp. Interestingly, the clones obtained from the kidney tissue of the second patient with PML (GU) contained a tandem triplication of 95 bp with a 32-bp deletion in the middle repeat, an arrangement similar to that found in the BKV

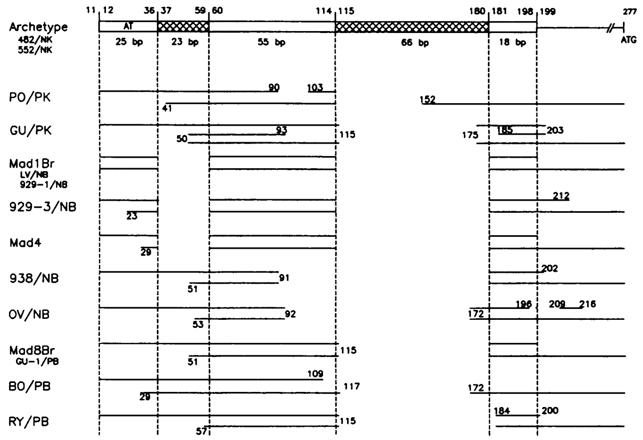

FIG. 3. Comparison of the regulatory regions of JCV variants isolated from PML- and non-PML-affected tissues. The structure of the archetype JCV(MY) regulatory region (72) is shown at the top of the diagram. Independently isolated clones having the same structural arrangement are indicated in smaller type below each listing. The regulatory regions of all additional variants are shown as lines; parallel lines for a single variant indicate a repeated sequence, and gaps in the lines indicate a deletion relative to the archetype sequence. The endpoints of each repeat element are shown as nucleotide numbers (archetype numbering) at the ends of the solid lines. The 23- and/or 66-bp blocks (hatched boxes; TAGGGAGGAG CTGGCTAAAA CTG and AGAGGGAGCC CTGGCTGCAT GC CACTGGCA GTTATAGTGA AACCACTCCC ATAGTCCTTA ATCACA, respectively) present in archetype are absent in many JCV variants, whereas the 55- and 18-bp blocks (GATGGCTGCC AGCCAAGCAT GAGCTCATAC CTAGGGAGCC AACCAGC AGA CAGCC and AGTAAACAAA GCACAAGG, respectively) are frequently conserved. The box labeled AT (25 bp; CTGTA TATAT AAAAAAAAGG GAAGG) contains the TATA element. The vertical dashed lines indicate the boundaries of each block in the variant sequences; the starting and ending nucleotide numbers of each block are indicated at the tops of the vertical lines on either side. The ATG to the right of the diagram represents the initiation codon for agnoprotein. A slash after the patient designation denotes a clone obtained from PCR products. Sequence variations among archetypes MY, CY, 482/NK, and 552/NK occur at positions 107*, 159*, 217*, 218, and 223 (* denotes a hypervariable position identified previously in archetype; 72). Nucleotides at these positions in MY, CY, 482/NK, and 552/NK are respectively A, T, T, and T (position 107); A, C, A, and C (position 159); A, G, G, and A (position 217); G, G, T, and G (position 218); and C, C, -, and C (position 223). In the sequence of a clone derived from the kidney of a PML patient (PO/PK), a single nucleotide, which is not found in the archetype sequence, immediately follows position 114 of each repeat (C after repeat 1 and A after repeat 2). The following abbreviations were used to designate the sources of the isolates: PB and PK for brain and kidney, respectively, from patients with PML; NB and NK for brain and kidney, respectively, from patients without PML. This figure was adapted from one by Yogo et al. (72).

(Dun) regulatory region (59). The regulatory regions of the kidney and brain variants of JCV in patient GU differ but are clearly related. Both have the characteristic deletion and amplification of promoter-enhancer sequences seen in PML-affected brain isolates. This is the first time two different "PML-type" clones have been identified directly in the tissues of a single individual. There has been one other report of isolating JCV from both the brain (GS/B) and kidney (GS/K) of a single patient with PML (41), but in that case, a strain resembling archetype JCV was identified in the kidney.

To check the fidelity of the PCR amplification, brain tissue samples from which JCV(Mad8Br) was originally isolated were obtained (26). Following amplification with JRR 1 and 2, multiple clones were derived; their sequences precisely matched that previously published for JCV(Mad8Br) (Fig. 3).

Twenty-one normal and PML-affected brain specimens were also examined by using BKV-specific primers and probes that were known to amplify sequences from five BKV variants [BKV(AS), BKV9, BKV(Dun), BKV(WT), and BKV(DB); data not shown]. Only one non-PML-affected brain sample in one experiment gave a positive signal following Southern blotting and hybridization with a BKV-specific oligonucleotide probe (data not shown). Three additional experiments failed to repeat this result.

The brain tissue samples which showed evidence by PCR of JCV infection (Table 2) were examined by immunocytochemical methods for the presence of JCV T antigen and by in situ hybridization techniques for the presence of JCV DNA. No evidence of active JCV infection was obtained by either method. The examination of hematoxylin-eosin-stained sections from these tissues showed no evidence of demyelinated foci or other pathological changes characteristic of PML.

DISCUSSION

It is well established that JCV is distributed in several tissues of patients with PML (14, 27, 32). While similar studies with non-PML-affected patients have not been as extensive, there is ample evidence that JCV persists in the kidneys of many of these individuals (10) and is occasionally released into their urine (3, 17, 34, 43, 50, 71, 72). A number of groups have also looked for JCV in the brains of non-PML-affected individuals (9, 10, 15, 31, 46, 64), but their findings have usually been negative or inconclusive. However, Mori et al. (47) have found evidence of JCV DNA and late proteins in glial cells associated with small demyelinated foci in three elderly patients. The highly sensitive and specific nature of the PCR technique has allowed us to examine the possibility that JCV might be present at levels too low to be detected by immunocytochemistry or in situ hybridization. Using the PCR approach, we have successfully amplified and identified JCV DNA in the brain and kidney tissues of individuals who died from causes unrelated to neurological complications. Although attempts to estimate the number of viral genomes present in each sample were not made, the strength of the signal obtained suggested a low copy number, perhaps 10 or fewer molecules, in some of the tissue sections. Given that JCV may often be present at very low levels and that even in PML-affected brain tissue the virus may have a focal distribution, it was not surprising that some sections of infected non-PML-affected brain failed to yield a positive signal. It is unlikely that this failure was due to the presence in the tissues of soluble inhibitors of the PCR; when tissue samples that consistently tested negative for viral sequences were "spiked" with recombinant JCV DNA, no inhibition of amplification was observed (data not shown).

Extensive precautions were taken in this study to reduce the possibility of generating false-positive reactions. Through the use of multiple primer and probe combinations complementary to different regions of the JCV genome, positive signals produced by cross hybridization with cellular or related viral (e.g., BKV) sequences were avoided. Furthermore, the identification of unique strains of JCV by sequence analysis (Fig. 3) provided strong evidence that positive signals did not represent contamination of the tissue samples with laboratory strains of virus. In two instances, however, the viral DNA obtained from non-PML-affected brain samples (from patients LV and 929) was identical to that of Mad1, the strain used as a positive control (pMad1-TC) in these experiments and the predominant strain studied in our laboratories. When primers that flanked the JCV-pBR322 junction of pMad1-TC were used, contamination of brain tissue sections from patients LV and 929 and from 13 other patients could be ruled out; potential contamination of a single sample (from patient 736) was detected (Table 2). It was also noted that while two of the three cloned regulatory regions of patient 929 had a JCV(Mad1) sequence, a third clone had a precise deletion of the distal TATA box found in JCV(Mad1) which yielded a variant similar to JCV(Mad4). Major et al. (42) have reported the recovery of JCV(Mad4) from the tumor cells of a monkey inoculated with JCV-(Mad1), suggesting that the latter variant may have rearranged to yield the former. The present observations are consistent with the possibility that the third 929 clone arose in the patient following a deletion event and was not a PCR or cloning artifact. This would argue, in turn, that the isolation of the two JCV(Mad1) clones was also biologically relevant.

The sequence data in Fig. 3 support the hypothesis that the JCV genome undergoes an adaptation process (14, 18–20, 44, 71, 72) in which the regulatory region of the archetype strain is rearranged by deletion and duplication events. It has generally been assumed that this process occurs during an immunocompromising event that contributes to the reactivation of a latent kidney infection. During increased replication of the viral genome, variants that are more active and that exhibit a new tissue tropism might arise. While reactivation of an infection appears to contribute to the process of adaptation, it is also important to consider what happens during a primary infection. Results from the present study indicate that at least two strains of JCV become established in the non-PML-affected patient, i.e., archetype in the kidney and PML type in the brain. If the viral genome is rearranged during active replication at the primary site of infection, only archetype becomes established in the kidney. In the brain, the PML-type variant might have a replicative advantage and establish a multifocal infection prior to the onset of an immune response that then limits the infection. Alternatively, the archetype variant might infect both organs prior to rearrangement, with adaptation occurring in the brain during subsequent growth and development of the host. It will be important in future experiments to investigate the effects of the deletion-duplication event that occurs in the archetype genome during adaptation. It appears especially relevant that nearly all JCV brain variants have lost most of the 66-bp block (Fig. 3), a sequence which shares homology with a negative regulatory element in BKV (25).

Transport of JCV to the brain presumably occurs via a hematogenous route, and the detection of JCV DNA and proteins in B lymphocytes of the bone marrow (32) supports this concept. This does, however, raise the possibility that the positive signals seen in non-PML-affected brain samples are due to the presence of infected lymphocytes in the lumina of cerebral vessels or of free virus in the circulation at the time of death. Although our data do not rule out this possibility, two lines of evidence argue against it. First, since BKV, like JCV, can reactivate in the kidney under conditions of immunosuppression, it also might appear in the circulation around the time of death. Thus, detection of BKV DNA in the circulation, and therefore apparently in the brain samples, might be expected as well. Yet, of 21 brain sections tested, only 1 exhibited a positive signal for BKV; additional experiments have failed to repeat even this result. Second, the failure to identify JCV archetype in brain tissue rules out the presence of normal kidney-type virus in vessels in these samples (as well as ruling out contamination of our samples by urinary virus acquired during handling) as an explanation of these results. We interpret these data to indicate that JCV is present within the brains of non-PML-affected patients and is not merely a passenger of circulating lymphocytes in the plasma.

If the JCV DNA sequences we have detected in brains without known neurological disease truly represent a latent infection, one would not expect to find viral proteins or replicating viral DNA in the samples. We therefore tested the tissues containing JCV DNA sequences for the presence of the early protein, T antigen, by using a sensitive immunocytochemical method capable of detecting T antigen in vascular endothelial cells of JCV-infected hamster brain tissue (56). We also conducted in situ hybridization techniques which can detect replicating JCV DNA in glial cells of patients with PML (1, 32). Both approaches yielded negative results. These findings are consistent with a latent infection which remains quiescent even in aged individuals such as patients AE, RA, and 884. Our findings contrast with those of Mori et al. (47), who reported evidence of DNA replication by in situ hybridization and the presence of JCV capsid proteins in glial cells around very small demyelinated foci in the brains of aged individuals without known neurological disease. It should be noted that Heinonen et al. (30) recently reported that they were unable to repeat the work of Mori et al. (47).

The unexpected findings reported in this study may be especially relevant to our understanding of the pathogenesis of PML in patients with AIDS. Although PML was once considered a rare disease, its incidence has increased dramatically because of the AIDS epidemic (6). This increase may not be due simply to the immunosuppressive effects of an HIV-1 infection; there is evidence in vitro that the HIV *tat* protein can transactivate JCV transcription (62). Given that JCV may reside in the non-PML-affected brain (this study) and that HIV-1 infection involves the central nervous system in a high proportion of patients with AIDS (36, 69, 70), it is possible that HIV-1 can reactivate a latent JCV infection, thereby increasing the risk of clinical PML. Alternatively, should reactivation occur prior to entry of HIV-1 into the brain, replication of JCV might lead to the recruitment of HIV-1-infected macrophages and lymphocytes to this site, thereby potentiating the spread of infection (67, 68). In either case, the presence of this opportunistic pathogen in many normal brains may have untoward consequences for an individual should that person experience a severe immunocompromising event such as a superinfection with an activating retrovirus.

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